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ORIGINAL PAPER

# Immunomodulatory Properties of Highly Viscous Polysaccharide Extract from the Gagome Alga (*Kjellmaniella crassifolia*)

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**Abstract** Marine brown algae are rich in sulfated polysaccharides, which have the ability to form gels and viscous solution. Sulfated polysaccharides exhibit many biological activities; however, little is known whether the viscoelastic property in the polysaccharide extract is correlated with biological activities. We examined the immunomodulatory properties of highly viscous polysaccharide extract (HVPE) from Gagome *Kjellmaniella crassifolia* in a murine model, and the effects were compared with those of a less viscous polysaccharide extract (LVPE). HVPE or LVPE (10, 30, and 100 mg/kg/day) were orally administered to C57BL/6 mice for 14 days. Secretions of cytokine and IgA in Con A-stimulated spleen and Peyer's patch (PP) cells and phagocytic activity of peritoneal macrophages was determined. IFN-γ, IL-12, IL-6, and IgA secretions showed high levels in spleen cell cultures from mice administered HVPE, whereas these effects were diminished in the LVPE-administered mice. The phagocytic activity of peritoneal macrophages was enhanced by the continuous oral administration of HVPE, and these effects were higher than those of LVPE. Furthermore, an increase in IgA secretion by administration of HVPE was observed in Con A-stimulated PP cells. These results suggest that the polysaccharide extract from *K. crassifolia* has immunomodulatory activities, which depend on the viscosity.

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## Introduction

Many kinds of seaweeds are traditionally consumed in Asia, and they are generally considered to be safe, nutritious, and health-beneficial [1]. Gagome (*Kjellmaniella crassifolia*) is a brown alga distributed around the southern area of Hokkaido, the northern mainland of Japan. Recently, *K. crassifolia* has attracted a lot of attention from the industrial field because it contains much more fucoidan than other seaweeds [2] and its wáter extract shows very high viscoelasticity.

Fucoidan is a sulfated polysaccharide that consists of fucose, uronic acids, galactose, and xylose, and their proportions and linkage position vary according to the species [3]. It has been reported that fucoidan has numerous biological activities as an anti-coagulant, anti-tumor, and immunomodulatory agent [4–6]. There have been many reports concerning the relationship between the sulfate content of fucoidan and its biological activity [7]. On the other hand, the importance of its molecular weight has also been demonstrated; a lower molecular-weight fucoidan (4 kDa) promotes basic fibroblast growth factor-induced tube formation of endothelial cells [8]. In contrast, Shimizu et al. [9] reported that higher molecular-weight fucoidan (200–300 kDa), when orally administered, increased the ratio of cytotoxic lymphocytes in murine splenocytes. There may be some differences in these results because the structures of fucoidan vary with the species of brown algae and the extraction method used [10]. It is probable that the biological activities of fucoidan are dependent on various factors, such as sulfation degree, molecular weight, and structural backbone, including sugar composition and branching degree.

Some reports are available on the relationship between the structure and immunomodulatory properties of polysaccharides [11]; however, little is known about the relationship between its viscosity and immunomodulatory properties. We here focused on the highly viscous polysaccharides obtained from *K. crassifolia*. Recently, we have established the preparation of a highly viscous polysaccharide extract from *K. crassifolia*, mainly fucoidan [12]. The viscosity of fucoidan prepared by the commonly procedure is very low even if it's high concentration (relative viscosity of 10 mg/ml solution is less than 1.5). The Gagome water-extract has very high viscosity compared with other edible seaweeds, such as *Nemacystus decipiens* and *Sargassum horneri*, and this food characteristic is important to utilize Gagome as a food material. The viscosity was affected by temperature, pH, and coexisting salts, and these changes in viscosity were caused regardless molecular degradation of polysaccharides. The viscosity as well as molecular weight should be an important factor in the immunomodulatory properties. The objective of this study was to investigate the immunomodulatory properties of a highly viscous polysaccharide extract from *K. crassifolia*. The oral administration of viscous polysaccharides to mice for 14 days was performed. The effects of a highly viscous extract on phagocytic activity of macrophages, and cytokine and IgA secretions in Con A-stimulated spleen and Peyer's patch (PP) cells were examined and compared to those of a less viscous extract.

## Materials and Methods

### Preparation of Viscous Polysaccharide Extract from *K. crassifolia*

Artificially cultured *K. crassifolia* was harvested at the Esan area in Hakodate city (Hokkaido, Japan). The seaweed was air-dried, vacuum-packed, and then kept at 3–7 °C in a refrigerator until use. A viscous polysaccharide extract from *K. crassifolia* was prepared in the same manner reported previously [12]. Briefly, the dried *K. crassifolia* (10 g) was cut into fine pieces and suspended in distilled water (240 ml). The extraction was carried out at 20 °C for 24 h with shaking at 100 rpm. After centrifuging, the supernatant was dialyzed overnight against distilled water at 4 °C and lyophilized as a highly viscous extract (HVPE). A less viscous extract (LVPE) was prepared by heat treatment of the HVPE solution in a 60 °C water bath for 1 h.

### Physical and Chemical Properties of Polysaccharides

The viscosity of polysaccharide solutions was determined using an Ostwald viscometer at 20±0.5 °C. The solution viscosity was expressed as the relative viscosity against water. The total sugar content was determined by the phenol-sulfate method [13] using fucoidan as a standard. Fractionation and quantification of fucoidan, alginate, and laminaran were conducted in the same manner reported previously [12]. The sulfate content was determined by the rhodizonate method [14] after hydrolysis with 1 M HCl for 5 h. The amount of reducing end group was determined by the Somogyi–Nelson method [15]. Ash was measured after burning the sample overnight at 550 °C.

### Oral Administration and Isolation of Immune Cells

C57BL/6 mice (male, 5 weeks old) were purchased from Charles River Japan (Yokohama, Japan). These mice were treated according to the guidelines for Animal Experimentation at Hokkaido University. HVPE and LVPE were dissolved in distilled water at concentrations of 1, 3, and 10 mg/ml, and the solution viscosity was 2.9, 12.2, and 462.7 for HVPE and 2.6, 7.6, and 55.6 for LVPE, respectively (Table 1). These solutions were administered orally to mice by intragastric intubation at 10 ml/kg body weight (corresponding to 10, 30, or 100 mg/kg/day) in distilled water once a day for 14 consecutive days. Control mice were also given distilled water on the same schedule. On day 15, the mice were sacrificed by $CO_2$ asphyxiation. Spleens and small intestines were aseptically removed. Single-cell suspension of spleen cells was prepared by passing homogenized spleens through a nylon mesh and treating with 0.83% ammonium chloride solution to remove red blood cells. PP was dissected from the small intestines, and single-cell suspension of PP cells was prepared by digesting with 125 U/ml type I collagenase containing 10 U/ml DNase. Peritoneal macrophages were harvested by lavaging the peritoneal cavity of mice with cold PBS. The collected cells were seeded into cell culture dishes, and incubated at 37 °C for 2 h to allow them to adhere. Non-adherent cells were removed by washing with PBS twice, and the adhesive cells were used as peritoneal macrophage.

### Measurement of Cytokine and IgA Levels

The harvested spleen and PP cells were treated with 1 μg/ml Con A, a T lymphocyte-specific mitogen, and then incubated at 37 °C. After 72 h and 120 h, the levels of cytokine and IgA, respectively, in the cell supernatant were measured by sandwich ELISA. For cytokine measurement, rat anti-mouse IFN-γ, IL-12, and IL-6 antibodies, biotinylated rat anti-mouse IFN-γ, IL-12, and IL-6 antibodies, and avidin-HRP conjugate was used. For IgA measurement, goat anti-mouse IgA antibody and goat anti-mouse IgA peroxidase conjugate was used, all of which were purchased from Pierce (Rockford, IL).

**Table 1** Chemical composition and solution viscosity of highly viscous polysaccharide extract (HVPE) and less viscous polysaccharide extract (LVPE)

| | Lyophilized powder (%, w/w) | | | | | | | Viscosity of 3 mg/ml solution |
|---|---|---|---|---|---|---|---|---|
| | Total sugars | Fucoidan | Alginate | Laminaran | Sulfate group ($SO_3Na$) | Reducing end group | Ash | |
| HVPE | 60.6 | 51.1 | 5.2 | 2.6 | 16.9 | 1.53 | 16.3 | 12.2 |
| LVPE | 62.4 | 48.0 | 5.7 | 1.7 | 17.5 | 1.57 | 17.8 | 7.6 |

### Macrophage Phagocytosis Assay

Peritoneal macrophages ($1 \times 10^6$ cells/ml) were incubated with heat-killed fluorescein-labeled *Escherichia coli* (K-12 strain) BioParticles (Molecular Probes, Eugene, OR) at 37 °C for 2 h according to the supplier's protocol. After washed with PBS, and extracellular fluorescence was quenched with trypan blue. The fluorescence intensity was determined at 480 nm excitation and 520 nm emission using a fluorescence microplate reader. Phagocytic activity of the macrophage form the sample administered mice was calculated as the percentage of fluorescence intensity of macrophages from control mice.

### Statistical Analysis

Data are expressed as the mean ± S.D. Statistical differences were tested using the Tukey-Kramer multiple comparison test and Student's *t*-test.

## Results and Discussion

### Chemical Composition and Solution Viscosity

We prepared the viscous polysaccharides from *K. crassifolia*. Table 1 shows the chemical composition and solution viscosity of HVPE and LVPE. HVPE was composed of 51.1% fucoidan, which was a main component. There was no difference in the chemical composition during the heat-treatment for preparing LVPE. No difference in the number of reducing end group was found, which revealed that the decrease in viscosity induced by heat treatment was not caused by degradation of polysaccharides.

### Cytokines and IgA Production of Spleen Cells

Spleen plays a significant role in host defense as well, contributing to both cell-mediated and humoral immunity.

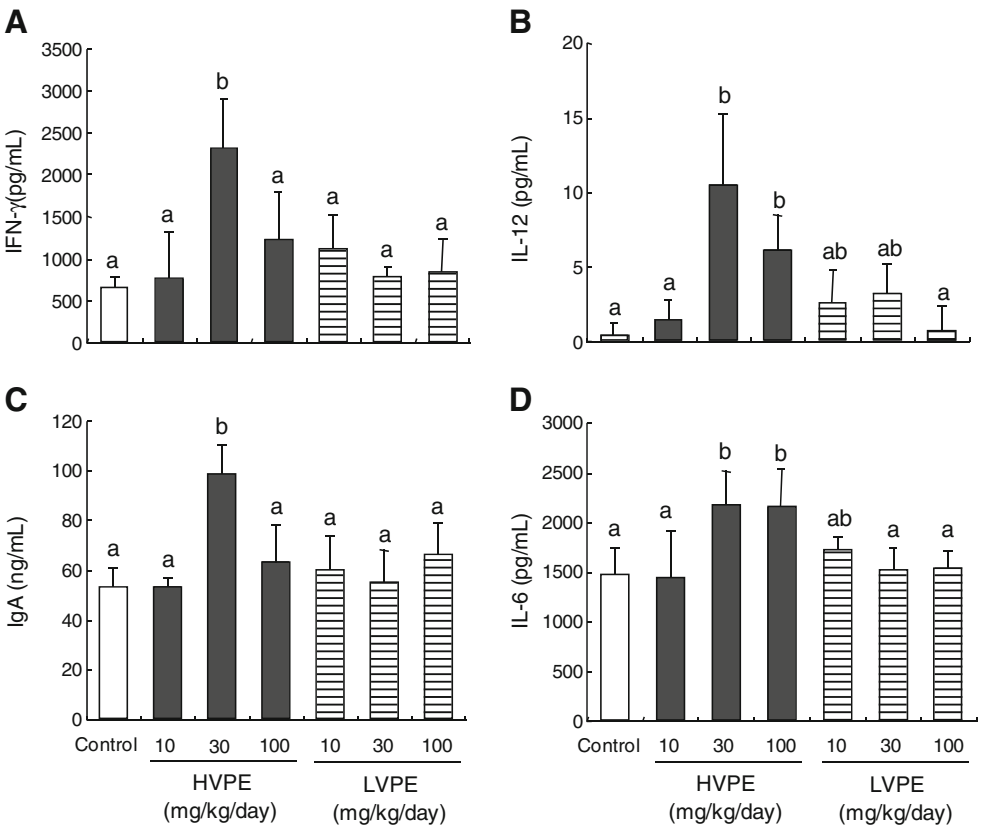


**Fig. 1** Production of IFN-γ (pg/ml) (**A**), IL-12 (pg/ml) (**B**), IgA (ng/ml) (**C**), and IL-6 (pg/ml) (**D**) in spleen cells. Values are the mean ± S.D. of five mice per group. Different letters indicate statistically significant differences ($P<0.05$) among different groups. HVPE , highly viscous polysaccharide extract; LVPE, less viscous polysaccharide extract

We first examined whether the viscosity in polysaccharides extracted from *K. crassifolia* affected the cytokine production of spleen cells. IFN-γ and IL-12 are critical for cell-mediated defense against various pathogens. As shown in Fig. 1a, the secretion of IFN-γ in the 30 mg/kg HVPE-administered mice group showed significantly high value rather than that of the control group. The high secretion levels of IL-12 were also observed in the 30 and 100 mg/kg HVPE-treated group (Fig. 1b). On the other hand, the increasing effect of LVPE on the secretion of the cytokines was slight and lower than that of HVPE. These results indicate the continuous administration of HVPE enhanced cell-mediated immunity in mice spleen cells.

Humoral immune responses are mediated by immunoglobulins and protect us from pathogen infections and antigen sensitization. To examine the effect of the viscous polysaccharides on humoral immunity, IgA concentration in the spleen culture supernatants were measured by ELISA (Fig. 1c). The increase in IgA production was observed in the 30 mg/ kg HVPE-administered group and the IgA level was 1.9-fold higher than that of the control group. In contrast, a significant increase in IgA productivity was not observed in the LVPE-administered group. Similar results were found in the production of IL-6 (Fig. 1d), which is an upregulation factor for B-cell activation and differentiation into IgA-producing plasma cells [16]. These results suggest the effectiveness of HVPE for an enhancement of humoral immunity.

Even though the dosage of HVPE at 100 mg/kg was corresponded to the highest viscosity among all the samples tested, significant enhancement of cytokine and IgA secretions was not observed in this group, compared to 30 mg/kg HVPE-treated group. Therefore, it is possible that the enhanced effect of viscous polysaccharides from *K. crassifolia* on splenic immune response depends on their solution viscosity and there is optimal viscosity for immunomodulatory properties. According to Park et al. [17], oral administration of high molecular weight (100 kDa) fucoidans enhanced the production of IFN-γ in collagen-stimulated spleen cells, while low molecular weight (1 kDa) fucoidans had the opposite effect. This suggests that polyanionic structure of fucoidan, sulfated polymer of L-fucose, is expected to allow it to bind to a large number of receptors, thus exert biological activities. In the present study, LVPE did not show any enhancement of cytokine and IgA secretions, regardless of the dosage. According to our previous report [12] and Table 1, heat-induced decrease in the viscosity occurred in LVPE was not involved in the degradation of polysaccharide molecules and the lack of a sulfate group because the contents of the reducing end and sulfate groups remained unchanged during the heat treatment. Therefore, during the heat treatment, fucoidan molecules might be changed to some local folding or aggregation, which leads to burial of the active sites such as sulfated groups.

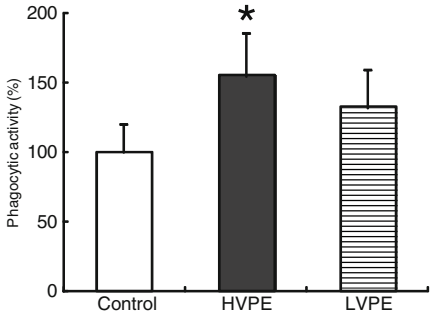


**Fig. 2** Effect of oral administration of HVPE or LVPE on the phagocytosis of peritoneal macrophages. Values are the mean ± S.D. of five mice per group. *$P<0.05$ versus control group

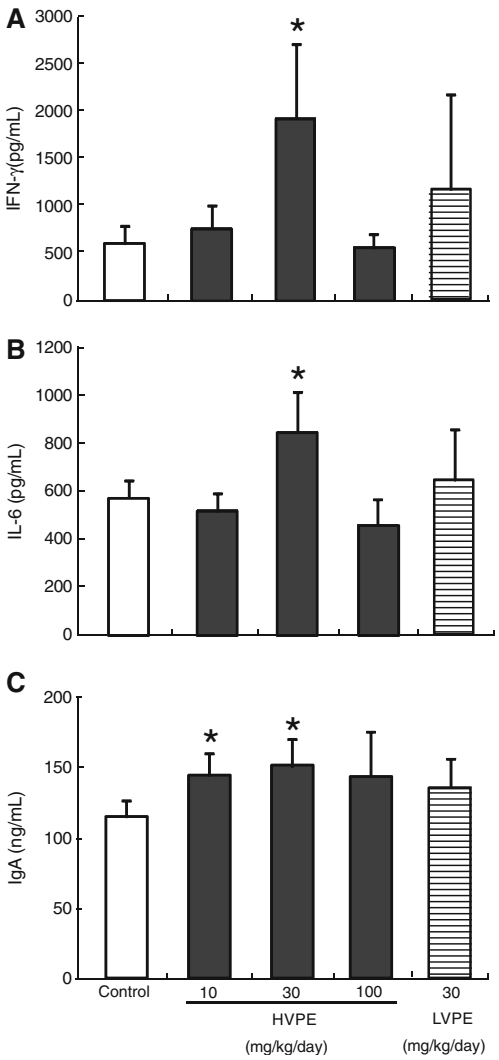


**Fig. 3** Production of IFN-γ (pg/ml) (**A**), IL-12 (pg/ml) (**B**), and IgA (ng/ml) (**C**) in Peyer's patch (PP) cells. Values are the mean of five mice per group ± S.D. *$P<0.05$ versus control group

### Phagocytic Activities of Peritoneal Macrophages

Figure 2 shows the phagocytic activity of peritoneal macrophages from the mice after the 14 days-oral administration of HVPE or LVPE at 30 mg/kg. The phagocytic activity of peritoneal macrophages in HVPE and LVPE-treated group was higher compared to that of the control group, and the phagocytic activity increased more in the HVPE-administered mice than that in the LVPE-administered group. Thus, it is apparent that peritoneal macrophages of mice were enhanced by the continuous administration of Gagome extract, particularly HVPE.

IL-12 is mainly known to activate NK and T cells and induce IFN-$\gamma$ secretion, which then acts back to activate macrophages early in an immune response [18]. Therefore, the activation of macrophages by oral administration of HVPE could be due to the elevation of IL-12 in spleen cells shown in Fig. 1b. Macrophage phagocytosis is an important defense of the non-specific or innate immune system against invading pathogens [19]. Taken together, the present study suggests that viscous polysaccharide from Gagome could be useful as an immunotherapeutic agent for treating infectious disease.

### Cytokine and IgA Production of PP Cells

We further investigated the effect of the viscous polysaccharides on mucosal immune responses (Fig. 3). The secretion level of IFN-$\gamma$ and IL-6 in the HVPE-administered mice group at 30 mg/kg showed higher and different values from that of the control group. In contrast, no significant increase was observed in these cytokine levels at the 30 mg/kg of LVPE-administered group. Further, IgA levels increased more in the 10 and 30 mg/kg of HVPE-administered mice than in the LVPE-administered group and the control. These results demonstrate that immunomodulatory activities of Gagome polysaccharide extract depend on the viscosity. Oral administration of HVPE at 100 mg/kg, which corresponded to the highest solution viscosity, had lower immune enhancing effects on PP cells as well as spleen cells, compared to that of HVPE at 30 mg/kg. Our previous study [12] showed that highly viscous polysaccharide extract from *K. crassifolia* forms an extended conformation of fucoidan owing to the charge repulsion. Thus, it seems that polysaccharide solutions with extremely highly viscosity might form huge macromolecules and most of them pass straight through the intestine without stimulating PP cells, resulting in decrease in immune activation.

After oral administration of the HVPE solution (3 mg/ml) containing blue dextran to mice, the mobility of the intestine was measured. Although the detailed data are not shown here, the blue-stained HVPE solution reached the almost end point of intestine 60 min later and the content was still sticky. It appears that viscous polysaccharides can influence the gut immunity mediated by intestinal intraepithelial T lymphocytes and PP. Indeed, cytokine and IgA levels in PP cells significantly increased by oral administration of HVPE, as demonstrated in Fig. 3. Considering the results of this work, it is likely that the intact polysaccharide exerts an enhancing effect on gut-associated immunity without impairing its structures and conformations, which then affects the systemic immunity via lymph nodes and peripheral blood.

In conclusion, this study has shown that oral administration of Gagome polysaccharide extract promotes the development of cell-mediated and humoral immune response, and the viscosity of the extract appears to be an important factor in mediating these immunomodulatory properties. Further studies of the molecular actions of viscous polysaccharides would allow us to use this extract as a valuable supplement or medicine for the prevention of cancer, autoimmune, and infectious diseases.

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